

Interactions Between Anandamide and Corticotropin-Releasing Factor Signaling Modulate Human Amygdala Function and Risk for Anxiety Disorders: An Imaging Genetics Strategy for Modeling Molecular Interactions

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ABSTRACT

BACKGROUND: Preclinical models reveal that stress-induced amygdala activity and impairment in fear extinction reflect reductions in anandamide driven by corticotropin-releasing factor receptor type 1 (CRF₁) potentiation of the anandamide catabolic enzyme fatty acid amide hydrolase.

METHODS: Here, we provide clinical translation for the importance of these molecular interactions using an imaging genetics strategy to examine whether interactions between genetic polymorphisms associated with differential anandamide (*FAAH* rs324420) and CRF₁ (*CRHR1* rs110402) signaling modulate amygdala function and anxiety disorder diagnosis.

RESULTS: Analyses revealed that individuals with a genetic background predicting relatively high anandamide and CRF₁ signaling exhibited blunted basolateral amygdala habituation, which further mediated increased risk for anxiety disorders among these same individuals.

CONCLUSIONS: The convergence of preclinical and clinical data suggests that interactions between anandamide and CRF₁ represent a fundamental molecular mechanism regulating amygdala function and anxiety. Our results further highlight the potential of imaging genetics to powerfully translate complex preclinical findings to clinically meaningful human phenotypes.

Keywords: Amygdala, Anxiety, CRHR1, FAAH, Habituation, Imaging genetics

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Converging evidence implicates endocannabinoid (eCB) signaling in the regulation of stress and anxiety, which may emerge, at least in part, through eCB modulation of amygdala output (1,2). Specifically, binding of the eCB ligand anandamide (AEA) to the cannabinoid type 1 receptor in the basolateral amygdala provides inhibitory tone through which multiple output processes, including fear extinction and anxiety, are modulated (1,3). In turn, the magnitude of this inhibitory tone is regulated by levels of fatty acid amide hydrolase (FAAH), the primary catabolic enzyme of AEA, with relatively decreased FAAH and subsequently increased AEA facilitating fear extinction and reducing anxiety-related behavior through maintenance of inhibitory tone (1,3,4).

Studies in rodents have revealed that chronic and acute stress increase FAAH activity resulting in reduced AEA signaling and diminished inhibitory tone and subsequently increased amygdala output including activation of the hypothalamic-pituitary-adrenal (HPA) axis and expression of anxiety-like behaviors (3,5–8). A recent study suggests that stress-related reductions in AEA are driven by corticotropin-releasing factor (CRF) release within the basolateral amygdala (9). Specifically,

antagonism of the corticotropin-releasing factor receptor type 1 (CRF₁) prevents stress-induced increases in FAAH activity; reciprocally, FAAH inhibition prevents CRF-mediated activation of the HPA axis and increased anxiety.

Here, we employ an imaging genetics strategy to model the effects of interactions between AEA and CRF signaling on human amygdala function and anxiety disorder diagnosis in 661 young adults. First, we modeled variability in AEA inhibitory tone through a nonsynonymous single nucleotide polymorphism (SNP) within the human *FAAH* gene (rs324420). This C to A polymorphism (C385A) results in a proline to threonine substitution at codon 129 (Pro129Thr), with reduced *FAAH* expression associated with the 385A allele (10,11). We have previously reported decreased threat-related amygdala reactivity in carriers of the 385A allele (12), reflective of relatively increased temporal habituation of amygdala activity to threat-related stimuli (4). Consistent with these data, a recent study has directly linked the 385A allele to potentiated fear extinction in humans, as well as a mouse knockin model of the *FAAH* C385A polymorphism (13). Secondly, we modeled variability in CRF signaling using a polymorphism within the human CRF₁ gene (*CRHR1*, rs110402).

This intronic A to G SNP has been associated with individual differences in HPA axis function as well as stress-related risk for depression (14–17). Critical to our current study, the A allele has been associated with elevated cortisol reactivity to an acute stressor suggesting greater activation of the HPA axis in A allele homozygotes [(18) but see also (19)].

By testing the effects of interactions between *FAAH* rs324420 and *CRHR1* rs110402 polymorphisms on amygdala activity, we were able to model parallel molecular interactions between AEA and CRF signaling recently demonstrated to modulate stress-related amygdala function in rodents (Figure 1). We hypothesized that relatively increased CRF signaling would be associated with blunted habituation of the basolateral amygdala specifically in individuals with relatively high AEA-mediated inhibitory tone (i.e., *FAAH* 385A allele carriers who are also *CRHR1* A allele homozygotes). Further, we examined if individual differences in amygdala habituation indirectly link this genetic background to the presence of an anxiety disorder. We focused on amygdala habituation, as opposed to activity, in light of evidence that reductions in amygdala activation are associated with successful fear extinction (20,21) and that decreased amygdala habituation is associated with psychopathology characterized by anxiety and excessive fear (22–25). A focus on habituation is further consistent with knockout and pharmacologic manipulation studies in rodents suggesting that eCB signaling is critical for fear extinction, but not conditioning (1,4,26), and prior evidence that genetic variation in the eCB system is associated with amygdala habituation in humans (1,27).

METHODS AND MATERIALS

Participants

Neuroimaging and genetic data were available from 726 participants who completed the ongoing Duke Neurogenetics Study by January 6, 2014. All participants provided written informed consent in accordance with Duke University guidelines and were in general good health. Study exclusion criteria included 1) medical diagnosis of cancer, stroke, diabetes requiring insulin treatment, chronic kidney or liver disease, or lifetime psychotic

symptoms; 2) use of psychotropic, glucocorticoid, or hypolipidemic medication; 3) conditions affecting cerebral blood flow and metabolism (e.g., hypertension); and 4) contraindications to magnetic resonance imaging scanning. DSM-IV Axis I and select Axis II (i.e., antisocial personality disorder and borderline personality disorder) were assessed with the electronic Mini-International Neuropsychiatric Interview (28) and Structured Clinical Interview for DSM-IV Axis II Personality Disorders (29). As the Duke Neurogenetics Study seeks to capture broad variability in psychiatrically relevant behavioral phenotypes (e.g., depression, anxiety), participants meeting diagnostic criteria for disorders other than psychosis were included in the study (Supplemental Table S1).

Of the 726 participants, 67 were excluded due to scanner-related artifacts in functional magnetic resonance imaging (fMRI) data ($n = 6$), incidental structural brain abnormalities ($n = 2$), a large number of movement outliers in fMRI data ($n = 21$; see data quality control procedures below), scanner malfunctions ($n = 2$), inadequate signal in our amygdala regions of interest (ROIs) ($n = 14$; see coverage description below), poor behavioral performance ($n = 20$; accuracy lower than 75%), incomplete fMRI data collection ($n = 1$), and failed genotyping data ($n = 1$). The final sample reported includes 661 total participants (age = 19.64 ± 1.24 ; 293 male participants; 121 with at least one DSM-IV Axis I disorder including bipolar [$n = 11$], generalized anxiety [$n = 12$], panic [$n = 6$], agoraphobia [$n = 10$], obsessive-compulsive disorder [$n = 6$], social anxiety [$n = 6$], alcohol abuse [$n = 44$], alcohol dependence [$n = 29$], cannabis abuse [$n = 14$], and cannabis dependence [$n = 8$]; 311 European Americans, 73 African Americans, 173 Asians, 40 Latino/as, and 64 multiracial or other).

Genotyping

Genomic DNA was isolated from saliva collected using Oragene DNA self-collection kits (DNA Genotek, Inc., Ottawa, Ontario, Canada) customized for 23andMe (Mountain View, California) (www.23andme.com). DNA extraction and genotyping were performed by the National Genetics Institute, a Clinical Laboratory Improvement Amendments certified clinical laboratory and subsidiary of the Laboratory Corporation of America. Custom Illumina BeadChip arrays were used to provide genome-wide data from which the following SNPs were extracted: *FAAH* rs324420 and *CRHR1* rs110402. SNPs were coded according to minor allele carrier status due to low numbers of minor allele homozygotes in cells—*CRHR1* G carriers ($n = 415$): *CRHR1* heterozygotes ($n = 277$) and *CRHR1* G homozygotes ($n = 138$) and *CRHR1* A homozygotes ($n = 246$); and *FAAH* A carriers ($n = 249$): *FAAH* heterozygotes ($n = 229$) and *FAAH* A homozygotes ($n = 20$) and *FAAH* C homozygotes ($n = 412$). The genotyping rate of rs110402 was 99.9% and Hardy Weinberg equilibrium criteria were met (all $ps > .22$) within each self-reported ethnicity subsample (Supplemental Table S2). The genotyping rate of rs324420 was 100% and was within Hardy Weinberg equilibrium in each self-reported ethnicity subsample (all $ps > .11$). To account for differences in ancestral background, ancestrally informative principal components were generated from eigenstrat v5.0.1 (30). K means cluster plotting and visual inspection of the top 10 components revealed that the top 5 principal components

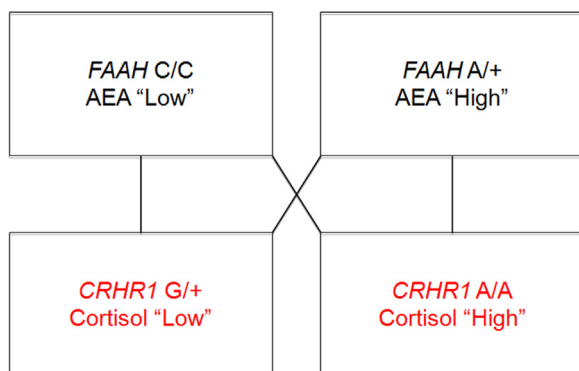


Figure 1. Modeling molecular interactions between anandamide (AEA) and corticotropin-releasing factor signaling. Imaging genetics strategy for modeling variability in AEA inhibitory tone and corticotropin-releasing factor signaling using interactions between *FAAH* rs324420 and *CRHR1* rs110402 genotypes.

accounted for the various subgroups within our study population (Supplemental Figure S1).

fMRI Paradigm

Our widely used amygdala activity paradigm consists of four face-matching task blocks interleaved with five shape-matching control blocks (31,32). During face-matching task blocks, participants view a trio of faces expressing angry, fearful, surprised, or neutral emotions (33) and select one of two faces presented on the bottom that match the target face presented on the top. Each expression-specific block (e.g., fearful facial expressions only) consists of six individual trials, with stimuli balanced for gender. Each of the six face trios is presented for 4 seconds with a variable interstimulus interval of 2 to 6 seconds (mean = 4 seconds); total block length is 48 seconds. During shape-matching control blocks, participants view a trio of geometric shapes (i.e., circles, horizontal and vertical ellipses) and select one of two shapes presented on the bottom that match the target shape presented on the top. Each control block consists of six different shape trios, each presented for 4 seconds (interstimulus interval = 2 seconds), making a total block length of 36 seconds. All blocks are preceded by a brief instruction (Match faces or Match shapes) lasting 2 seconds. The total paradigm is 390 seconds in duration. Reaction times and accuracy are recorded through a magnetic resonance compatible button box.

fMRI Acquisition Parameters

Participants were scanned at the Duke-University of North Carolina at Chapel Hill Brain Imaging and Analysis Center using two identical GE MR750 3T scanners (General Electric Healthcare, Little Chalfont, United Kingdom) equipped with high-power high-duty cycle 50-mT/m gradients at 200 T/m/s slew rate, and an eight-channel head coil for parallel imaging at high bandwidth up to 1 MHz. Blood oxygen level-dependent fMRI data were acquired using a semiautomated high-order shimming program to ensure global field homogeneity. A series of 34 interleaved axial functional slices aligned with the anterior commissure-posterior commissure plane were acquired for full-brain coverage using an inverse-spiral pulse sequence to reduce susceptibility artifact (repetition time/echo time/flip angle = 2000 ms/30 ms/60°; field of view = 240 mm; $3.75 \times 3.75 \times 4$ mm voxels [selected to provide whole-brain coverage while maintaining adequate signal-to-noise and optimizing acquisition times]; interslice skip = 0). Four initial radiofrequency excitations were performed and subsequently discarded to achieve steady-state equilibrium. To allow for spatial registration of each participant's data to a standard coordinate system, high-resolution three-dimensional structural images were acquired in 34 axial slices coplanar with the functional scans (repetition time/echo time/flip angle = 7.7 s/3.0 ms/12°; voxel size = $.9 \times .9 \times 4$ mm; field of view = 240 mm, interslice skip = 0).

fMRI Processing and Analysis

Whole-brain image analysis was completed using the general linear model of Statistical Parametric Mapping 8 (SPM8; Wellcome Trust Centre for Neuroimaging, Oxford, United

Kingdom) (<http://www.fil.ion.ucl.ac.uk/spm>). Images for each participant were first realigned to the first volume in the time series to correct for head motion before being spatially normalized into the standard stereotactic space of the Montreal Neurological Institute template using a 12-parameter affine model. Data were then smoothed to minimize noise and residual differences in gyral anatomy with a 6-mm full width at half maximum Gaussian filter. Voxelwise signal intensities were ratio normalized to the whole-brain global mean. The ARTifact Detection Tool (34) was used to generate regressors accounting for volumes associated with large motion (i.e., $>.6$ mm relative to the previous time frame) or spiking artifacts (i.e., global mean intensity 2.5 standard deviations from the entire time series). Participants for whom more than 5% of acquisition volumes were flagged by the ARTifact Detection Tool ($n = 21$) were removed from analyses. An ROI mask (Automated Anatomical Labeling template) from WFU PickAtlas (35) was used to ensure adequate blood oxygen level-dependent signal across the amygdala. Participants who had less than 90% coverage of the amygdala ($n = 14$) were excluded from analyses.

Linear contrasts using canonical hemodynamic response functions were then used to estimate temporal habituation as the linear decrease over successive face-matching task blocks (i.e., block 1 > 2 > 3 > 4) within right and left basolateral amygdala subregions defined using anatomical probability maps (36). The basolateral amygdala was selected as the ROI due to the effects of eCB signaling documented in this region in rodent models (1,3).

Individual contrast images (i.e., weighted sum of the beta images) were used in second-level random effects models accounting for scan-to-scan and participant-to-participant variability to determine mean habituation responses using one-sample *t* tests. A voxel-level statistical threshold of $p < .05$, familywise error corrected for multiple comparisons across the bilateral ROIs, and a cluster-level extent threshold of 10 contiguous voxels was applied to these analyses. Parameter estimates from maximal voxels in the right and left clusters exhibiting a main effect for the habituation contrast were extracted using the VOI tool in SPM8 and exported for regression analyses in SPSS version 20 (IBM Inc., Armonk, New York). Extracting parameter estimates from clusters exhibiting a main effect of condition, rather than those specifically correlated with our independent variables of interest, prevents correlation coefficient inflation that may result when an explanatory covariate is used to select a ROI. This conservative strategy has been implemented successfully in our prior studies (37–39).

Statistical Analyses

Before analyses, all habituation values from the above analyses were winsorized to maintain variability but constrain the influence of extreme outliers (i.e., following data quality control procedures, outliers more than ± 3 SDs were set at ± 3 SDs from the mean for 10 values from the left basolateral amygdala [1.5%] and 5 values for the right [.76%]). Moderation analyses were conducted using linear regression using the PROC-ESS macro (40) in SPSS to test whether FAAH rs324420 × CRHR1 rs110402 genotype interactions predicted amygdala

habituation after accounting for independent main effects of each genotype, as well as covariates for participant gender and ancestry using principal component values. The interactions between all covariates and predictor variables (e.g., *FAAH* genotype × gender, *CRHR1* genotype × principal component 1) were included as additional covariates to better isolate the genotype interactions of specific interest (41). All predictor variables were mean centered before the computation of interaction terms. Significant interactions were probed using Johnson-Neyman post hoc analyses. As an extension of these primary analyses, we examined whether amygdala habituation was predictive of any DSM-IV anxiety disorder. Any significant associations were then tested for moderated mediation to assess whether the *FAAH* rs324420 × *CRHR1* rs110402 genotype interaction was indirectly associated with anxiety disorder diagnosis through blunted amygdala habituation. Covariates here were identical to those included in the *FAAH* rs324420 × *CRHR1* rs110402 genotype moderation analysis above.

RESULTS

As previously reported (1), there was significant temporal habituation of left and right basolateral amygdala activation across all participants (Figure 2). A significant *FAAH* rs324420 × *CRHR1* rs110402 genotype interaction predicted habituation in the left basolateral amygdala ($F_{1,639} = 7.39, p = .0067, \beta = -.167, \Delta R^2 = .011$) and right basolateral amygdala ($F_{1,639} = 7.46, p = .0065, \beta = -.142, \Delta R^2 = .011$) (see Supplemental Table S3 for full regression model). As predicted, post hoc tests demonstrated that individuals with a genetic background associated with relatively increased AEA inhibitory tone and increased CRF signaling (i.e., *FAAH* 385A allele carriers who were also *CRHR1* A allele homozygotes) exhibited the least amygdala habituation (Figure 3A, B). This group of individuals

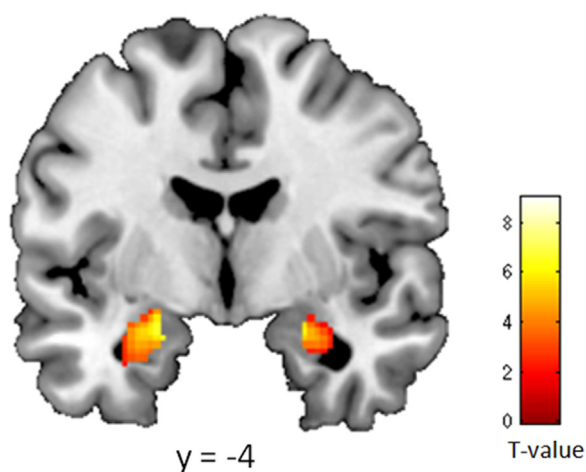


Figure 2. Basolateral amygdala habituation during face-matching task blocks. Overlay illustrating clusters in the right and left basolateral amygdala exhibiting significant temporal habituation modeled as the linear decrease over successive face-matching task blocks (i.e., block 1 > block 2 > block 3 > block 4). Statistics and coordinates of peak voxel within each significant region of interest. Left basolateral amygdala: $t = 9.00; x = -22, y = -8, z = -16$. Right basolateral amygdala: $t = 9.25, x = -22, y = -8, z = -16$. All $ps < .001$, familywise error < .05.

exhibited significantly blunted habituation in comparison to those with relatively increased AEA but decreased CRF (i.e., *FAAH* 385A allele carriers who were also *CRHR1* G allele carriers; left: $F_{1,241} = 13.22, p = .0003$; right: $F_{1,241} = 11.35, p = .001$), as well as those with relatively decreased AEA but increased CRF (i.e., *FAAH* C385 G allele homozygotes who were also *CRHR1* A allele homozygotes in the left basolateral amygdala, $F_{1,238} = 4.08, p = .045$, and a trend level non-significant difference in the right basolateral amygdala, $F_{1,238} = 3.28, p = .071$). Although not significant, potentially due to reductions in power, a similar pattern was observed in a subsample of European/European-American participants only ($n = 311$; left: $F_{1,304} = 3.22, p = .074$; right: $F_{1,304} = 3.36, p = .068$) (Supplemental Figure S2).

In our full sample, 36 participants met DSM-IV criteria for a past or present anxiety disorder (Supplemental Table S1). While there was no significant direct effect of *FAAH* rs324420 × *CRHR1* rs110402 genotype interactions on anxiety disorder diagnosis ($b = -2.76, p = .42$), there was a significant association between blunted left basolateral amygdala habituation and increased risk for an anxiety disorder ($b = -1.57, p = .02$). Moreover, a moderated mediation analysis indirectly linked the interaction between *FAAH* and *CRHR1* genotypes to diagnosis of an anxiety disorder through left basolateral amygdala habituation (.26; bootstrapped 95% confidence intervals [CI]: lower limit [LL]: .029, upper limit [UL]: .603) (Figure 3C). This moderated mediation was due to a conditional indirect effect wherein individuals with relatively increased AEA inhibitory tone and increased CRF signaling (i.e., *FAAH* 385A allele carriers who were also *CRHR1* A allele homozygotes) were more likely to have an anxiety disorder as a function of reduced amygdala habituation ($-.163$; bootstrapped 95% CI: LL: $-.414$, UL: $-.005$). No such effect was found for individuals with relatively increased inhibitory tone and decreased CRF signaling (i.e., *FAAH* 385A allele carriers who were also *CRHR1* G carriers; .099; bootstrapped 95% CI: LL: $-.008$, UL: .264). Neither left amygdala habituation nor interactive effects of *FAAH* rs324420 and *CRHR1* rs110402 genotype predicted alcohol use disorders (the most prevalent form of psychopathology in the sample) or the presence of any DSM-IV Axis I psychopathology (Supplement).

DISCUSSION

The present results uniquely extend recent observations in rodents that stress-induced interactions between AEA and CRF signaling modulate amygdala function associated with anxiety and fear extinction (9). In rodents, stress-induced CRF signaling via CRF_1 in the basolateral amygdala results in increased activity of *FAAH*. The increased activity of this catabolic enzyme subsequently results in decreased AEA and a loss of inhibitory tone necessary for reducing anxiety and maintaining fear extinction. Here, we demonstrate parallel effects in humans using two functional genetic polymorphisms to model variability in AEA inhibitory tone and CRF signaling. Specifically, we found the least temporal habituation of the basolateral amygdala, a neuroimaging correlate of fear extinction, in individuals who have relatively high AEA inhibitory tone (i.e., *FAAH* 385A allele carriers) and relatively high CRF signaling (i.e., *CRHR1* A allele homozygotes). Moreover, the

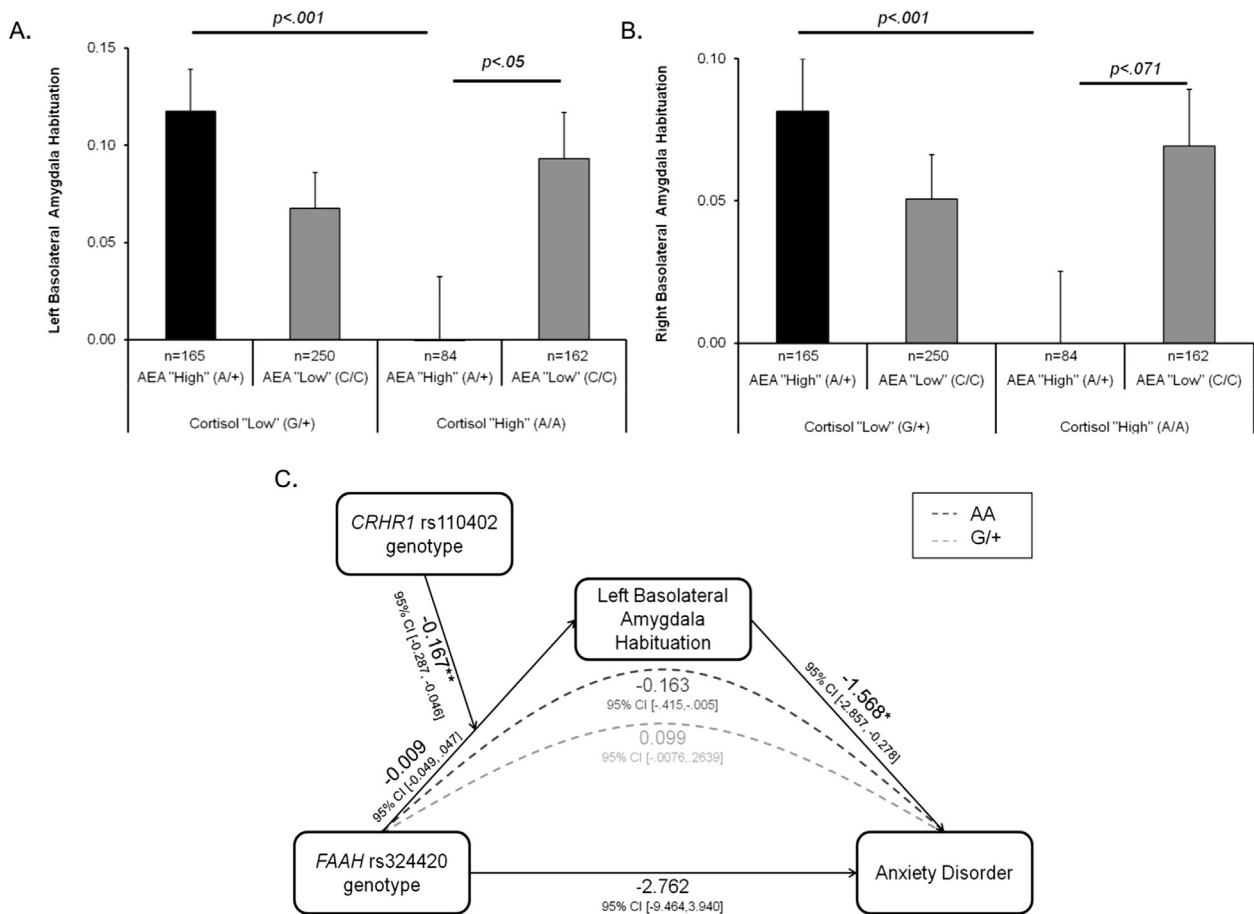


Figure 3. Genetic polymorphisms affecting anandamide (AEA) and corticotropin-releasing factor receptor type 1 signaling predict basolateral amygdala function, which indirectly mediates an increased risk for anxiety disorders. A significant interaction between *FAAH* rs324420 × *CRHR1* rs110402 genotypes predicts temporal habituation of the left (A) and right (B) basolateral amygdala. (A, B) Individuals with relatively increased AEA inhibitory tone and increased corticotropin-releasing factor signaling (i.e., *FAAH* 385A allele carriers who were also *CRHR1* A homozygotes) showed the least temporal habituation. Error bars indicate SEM. (C) Relatively reduced amygdala habituation mediated a significant increase in rates of anxiety disorders among individuals with relatively increased AEA inhibitory tone and increased corticotropin-releasing factor signaling (i.e., *FAAH* 385A allele carriers who were also *CRHR1* A homozygotes). Pathway coefficients represent unstandardized betas. * $p < .05$, ** $p < .01$. 95% CI, 95% confidence interval.

blunted habituation of the left amygdala in these individuals mediated a significantly increased risk for anxiety disorders. Although speculative, the laterality of this mediated risk may reflect the preferential contributions of the left amygdala to sustained evaluation of threat (42), which is a distinguishing feature of anxiety disorders (43).

Our study is not without limitations. Modeling variability in signaling pathways using functional genetic polymorphisms does not provide direct evidence for these interactions in humans; establishing functional correlates of our target polymorphisms through assays of circulating cortisol or AEA concentrations would underscore the accuracy of our model (44). The amygdala habituation phenotype we examined may be associated with fear extinction; for instance, during early extinction trials the amygdala is activated by stimuli previously conditioned to aversive outcomes, while in late extinction trials it is not (20,21). However, our task did not condition individuals to specific stimuli. Instead, it relied upon stimuli that have presumably been conditioned in everyday experience (e.g.,

facial expressions of fear). As such, amygdala habituation as presently measured is not a direct analog of traditional fear extinction (e.g., to a stimulus previously conditioned within the laboratory) [e.g., (25)]. Further, we did not collect behavioral measures of fear extinction (e.g., skin conductance response) (1,13), which may, speculatively mediate associations between amygdala habituation and anxiety disorder risk. The concurrent examination of neural and behavioral phenotypes in the context of imaging genetics research may provide clues to these relationships in future research.

The cross-sectional nature of our study is a further limitation regarding the contribution of the observed pathways to risk for anxiety disorders. Our moderated mediational model makes specific directional predictions regarding the link between amygdala habituation and psychopathology. While there is a robust but primarily nonhuman animal literature that is consistent with these directional assumptions, it is possible that alterations in habituation follow rather than precede the development of an anxiety disorder. Notably, however, blunted

amygdala habituation has been observed in at-risk children before the development of an anxiety disorder (24). Lastly, while the *FAAH* polymorphism investigated has been well characterized with regard to *FAAH* expression (10,11), the functional characterization of the *CRHR1* polymorphism is based upon previously reported associations with cortisol, a downstream consequence of CRF₁ activation (18), of which null reports exist (19) and which evidence suggests that childhood adversity may moderate (14).

Despite these limitations, our findings suggest conserved effects of interactions between AEA and CRF on amygdala function in rodents and humans and highlight the value of imaging genetics for translating preclinical findings to clinical phenotypes. These data are further useful for understanding how pharmacologic manipulation of the endocannabinoid system may be harnessed to treat anxiety and serve as a cautionary note on the potential importance of individual differences (45,46). Our data suggest that relatively increased AEA inhibitory tone moderates the anxiogenic effects of increased CRF signaling through attenuated amygdala habituation. As such, targeted facilitation of AEA inhibitory tone (through *FAAH* inhibition) may decrease anxiety and promote fear extinction in the absence of high CRF signaling but may have paradoxical effects in the presence of increased CRF signaling by pharmacologically increasing the dependence of amygdala regulation on AEA-mediated inhibitory tone. Alternatively, however, it is possible that enhanced anxiogenic effects of CRF in the context of high AEA may not simply be explained by overall levels but by the increased ability of CRF against this background to compromise AEA signaling by increasing *FAAH* activity. Thus, *FAAH* inhibition may be even more effective in the context of both high CRF₁ and AEA signaling, as it may prevent the anxiogenic effects of CRF by blocking the ability of CRF₁-stimulated increases in *FAAH* to inhibit AEA. Research in rodents showing that stress-induced anxiety and related phenotypes are prevented by pharmacologically inhibiting *FAAH* is consistent with this interpretation (3,5,9). Further pharmacogenetic research is necessary, however, to dissociate these proposed models and validate the efficacy of *FAAH* inhibitors as a potential therapeutic treatment approach.

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